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Analysis of Antibody Responses Against Coxsackie Virus B4 Protein 2C and the Diabetes Autoantigen GAD_{65}

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Type I diabetes mellitus results from the autoimmune destruction of insulin producing beta cells in the pancreas. Certain viral infections, especially those caused by coxsackie B viruses and related enteroviruses, have been associated with the development of type I diabetes. The sequence homology between the coxsackie B4 virus nonstructural protein 2C (CVB4 p2C) and the major diabetes autoantigen glutamic acid decarboxylase (GAD_{65}) provides a basis for the hypothesis of molecular mimicry. In this study, we investigated the prevalence of antibodies directed against nonstructural enterovirus proteins. In addition, a correlation of antibodies against CVB4 p2C and GAD_{65} was studied in diabetes patients and in healthy controls. Antibody reactivity against CVB proteins was detected by immunoprecipitation of [^{35}S]-methionine-labelled viral proteins and GAD_{65} antibodies were measured in a quantitative radio-immunoassay. It was shown that antibodies raised against the nonstructural proteins of CVB4 are very common in the population and a high degree of heterotypic cross-reactivity exists between different enterovirus types. CVB4 p2C-specific antibodies were not only detectable in GAD_{65} antibody-positive diabetes patients but also in GAD_{65} antibody-negative healthy blood donors. Furthermore, GAD_{65} antibodies could not be detected in p2C-positive subjects who had various enterovirus infections, indicating that an antibody response to CVB4 p2C does not necessarily induce a cross-reactive immune response against GAD_{65}. A correlation was not found between antibodies against GAD_{65} and p2C. ***J. Med. Virol. 59:256–261, 1999.***

KEY WORDS: type I diabetes mellitus; enterovirus; autoantibody; nonstructural proteins; molecular mimicry

INTRODUCTION

Coxsackie B virus infections have been associated with the development of type 1 diabetes mellitus. The association is based on virus isolations from newly diagnosed patients [Champsaur et al., 1982; Banatvala, 1987], induction of hyperglycaemia in animals by coxsackie B viruses (CVB) [Yoon et al., 1979; Szopa et al., 1990; See and Tilles, 1995], and sero-epidemiological studies in humans [Banatvala, 1987; Frisk et al., 1992]. Case-control studies have shown an increased prevalence and elevated levels of IgM antibodies to CVBs in newly diagnosed diabetes patients as compared with healthy controls, indicating a recent or ongoing infection at onset of the disease [Gamble et al., 1969; Frisk et al., 1992]. More evidence for a role of CVB infections in the disease process was obtained from prospective follow-up studies in Finland. During the preclinical phase, diabetes patients were more frequently infected with CVB than their matched controls [Hyöty et al., 1995; Lönnrot et al., 1998]. Even intrauterine exposure to the virus has been reported as a risk factor for development of diabetes mellitus [Dahlquist et al., 1995]. Moreover, patients seroconverted for islet cell autoantibodies during a CVB infection, suggesting that CVB infections triggers autoimmunity [Hiltunen et al., 1997].

Coxsackie B viruses belong to the family of enteroviruses that are common human pathogens that usually cause an acute infection. The clinical manifestations vary from asymptomatic infections to severe medical conditions such as paralysis, meningoencephalitis, and

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myocarditis [Melnick, 1996]. Whereas virus-specific T lymphocytes are activated during an infection [Graham et al., 1993], antibody responses are pivotal in the defence against enteroviruses and are responsible for clearance of the infection. Neutralising antibodies are directed against the capsid surface of the virus and are type specific. It is assumed that antibodies against nonstructural proteins of CVB are induced as well, but surprisingly little has been published on this subject [Ehrenfeld et al., 1995]. Nonstructural proteins are produced exclusively during replication of the virus and are released as a consequence of cell lysis of the infected cells.

Immune responses against nonstructural proteins of enteroviruses may play a role in type I diabetes mellitus. It has been suggested that a sequence similarity of the CVB4 protein 2C (CVB4 p2C) and a major diabetes autoantigen, glutamic acid decarboxylase (GAD_{65}), results in immunologic cross-reactivity, a process referred to as *molecular mimicry* [Kaufman et al., 1993]. GAD_{65} is a key target antigen in the pathogenic process of murine diabetes. In mice, GAD_{65}-specific reactivity coincides with the onset of insulitis, and it has been shown that diabetes can be prevented by tolerisation to GAD_{65} [Tisch et al., 1993; Tian et al., 1996]. One of the immunologic determinants of GAD_{65} is a peptide containing the region of similarity with p2C (PEVKEK) [Richter et al., 1994]. T cells responding to this mimicry sequence have been demonstrated in nonobese diabetes (NOD) mice and in patients with type 1 diabetes mellitus [Atkinson et al., 1994; Tian et al., 1994; Schloot et al., 1997]. Autoantibodies against GAD_{65} are present in the majority of newly diagnosed patients, and can be detected several years before clinical onset of the disease [Baekkeskov et al., 1990; Ellis and Atkinson, 1996]. It has been shown that antibodies induced by CVB4 p2C peptides cross-react with the homologous GAD_{65} peptides [Hou et al., 1994; Lönnrot et al., 1996], but the relevance for the pathogenesis of type 1 diabetes mellitus is disputable [Richter et al., 1994]. The prevalence of naturally induced antibodies against p2C has not yet been studied.

In this study, antibody prevalences were analysed against enterovirus nonstructural proteins in subjects who had various enterovirus infections, in healthy individuals, and in newly diagnosed type I diabetes mellitus patients. In addition, a correlation between antibodies against CVB4 p2C and GAD_{65} was investigated.

SUBJECTS AND METHODS

Subjects and Serum Samples

Convalescent serum samples were drawn from 26 individuals with a culture proven enterovirus infection. The viral infections comprised CVB2 (2), CVB3 (5), CVB4 (2), coxsackievirus A9 (CVA9) (2), ECHO virus 6 (EV6) (1), EV7 (1), EV9 (7), EV11 (2), EV25 (2), EV29 (1), and EV30 (1). Paired serum samples comprising an acute and a convalescent sample were collected from 8 additional patients. The samples were collected within a time interval of 10–20 days. Neutralising antibody titres were determined routinely and showed a significant increase in the convalescent serum samples. The patients experienced an infection caused by CVB3 (5), CVB4 (1), or EV11 (2) based on virus isolated from the throat. The sera were tested for the presence of antibodies against CVB structural and nonstructural proteins and GAD_{65} autoantibodies. Serum samples from 26 healthy blood donors were included in the study. Serum samples from 22 recently diagnosed type I diabetes mellitus patients (mean age 9 years) and samples from 5 nondiabetic siblings (mean age 12 years) were obtained. The samples were collected within a 3-month time period and were obtained from the same geographical region (the provinces Zeeland and Holland). All sera were tested for GAD_{65} antibodies and antibodies against CVB4 p2C as well as other nonstructural enterovirus proteins.

GAD_{65} Antibody Assay

Antibodies against GAD_{65} were measured by a validated radio-binding assay, performed as described previously [Petersen et al., 1994]. The results were correlated to standard negative and positive control sera and were expressed as GAD_{65} index. Antibody levels were shown as the median of a triplicate. GAD_{65} antibody positivity was determined as index > 0.05.

Viruses and Labelling of Viral Proteins

The CVB4 strain was a clinical isolate of which the homology with GAD_{65} had been established previously [Vreugdenhil et al., 1998]. CVB3, CVB4, and poliovirus Mahoney strain were cultured and propagated in Vero cells. Viral proteins were labelled with [^{35}S]-methionine as described previously [van Kuppeveld et al., 1997]. Extracts of either CVB3-, CVB4-, poliovirus- or mock-infected cells were prepared by incubation with a hypotonic buffer for 30 min at 37°C and three cycles of freezing and thawing. The amount of incorporated [^{35}S]-methionine was measured in a liquid scintillation counter. Protein labelling was analysed further by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, as described previously [van Kuppeveld et al., 1997]. Following this procedure, the virally infected cell extracts contained labelled proteins that were almost exclusively of viral origin.

Immunoprecipitation of Enterovirus Proteins

Antibodies against viral proteins were measured by immunoprecipitation of virally infected Vero cell lysates. Twenty microlitres of the serum samples were incubated overnight at 4°C with 200,000 counts per minute of [^{35}S]-methionine-labelled virally infected cell lysates in a total volume of 100 μl Tris-buffered saline (pH 7.5) complemented with 0.5% Tween-20 (TBS-T). The mixtures were then incubated with 14.3 mg of protein A-Sepharose 4B [Pharmacia Biotech, Uppsala, Sweden] per sample for 2 hr at 4°C. The precipitated proteins were washed five times with TBS-T and once with ice-cold H_2O and analysed by SDS-PAGE and au-

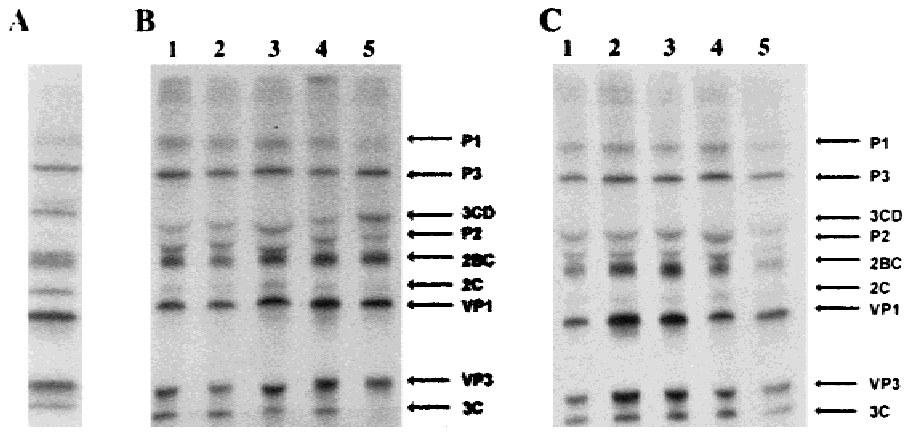

Fig. 1. Immunoprecipitation of coxsackie B4 virus (CVB4) proteins. **(A)** In vivo labelling of total CVB4 proteins. **(B)** CVB4 proteins immunoprecipitated by serum from patients with a recent enterovirus infection of different serotypes: **lane 1**, coxsackievirus A9 (CVA9); **lane 2**, ECHO virus 29 (EV29); **lane 3**, EV9; lane 4 and 5 CVB4. **(C) Lanes 1–5**, CVB4 protein precipitation by serum from healthy donors.

toradiography. The specificity of the method was confirmed by including serum obtained from a pathogen-free rabbit. In addition, we tested for nonspecific binding of the viral proteins to the Sepharose beads by protein precipitation in the absence of serum.

Rabbit Anti-p2C Serum

The 2C gene of CVB4 was cloned in the pGEX 4T2 vector and expressed as a glutathione S-transferase (GST) fusion protein (Promega, Madison, WI). After purification and cleavage of the GST fusion protein, recombinant p2C was used for rabbit immunisation. The rabbits were kept under pathogen-free conditions. The rabbit antiserum was tested for specificity by immunoblot. The anti-CVB4 p2C serum was used as a positive control of p2C precipitation.

CVB4 p2C Antibody Detection

P2C was translated in vitro and used for immunoprecipitation. The 2C gene was cloned into a pCITE-1 vector behind a T7 RNA polymerase promotor (Novagen, Madison, WI). In vitro translation reactions were carried out as described previously [van Kuppeveld et al., 1996]. Anti-p2B rabbit antiserum, kindly provided by K. Bienz, was used as a negative control.

RESULTS

Antibodies Against Coxsackie Virus B4 Proteins

Serum samples from 26 subjects with a recent, culture-proven enterovirus infection and from healthy donors were tested for antibody prevalence against CVB4 proteins labelled in vivo (Fig. 1A). Antibodies, present in the sera obtained from patients with a recent enterovirus infection, precipitated the whole range of CVB4 proteins: Nonstructural proteins, their precursor proteins, and capsid proteins were recognised (Fig. 1B). Antibodies against CVB4 proteins were also detected in serum samples from healthy donors, indicating that these donors had been exposed to enteroviruses previously (Fig. 1C). Some variation in antibody reactivity against the nonstructural protein p3C was observed. To investigate the presence of antibodies against nonstructural CVB proteins, we tested paired serum samples, that is, an acute and a convalescent sample, exposing a significant raise in CVB neutralising IgM antibodies. No difference in the overall pattern of precipitated proteins was observed between the acute (IgM negative) and the convalescent (high IgM titre) serum samples (Fig. 2).

Heterotypic Cross-Reactivity Between Different Enterovirus Types

The serum samples tested were obtained from patients with various CVA, CVB, and EV infections. All sera precipitated proteins of the CVB4 strain, irrespective of the viral type by which the patients were infected. In addition, serum samples were tested for precipitation of [^{35}S]- labelled extracts from CVB3-, CVB4-, poliovirus-, and mock-infected cells. Viral proteins from each of these enteroviruses were precipitated, whereas mock-infected lysates were not recognised (data not shown).

Antibodies Against GAD_{65} and CVB4 Viral Proteins

Twenty-two serum samples obtained from patients with type 1 diabetes mellitus were tested for GAD_{65} antibodies. Fifteen of the 22 patients and 1 of 5 siblings were GAD_{65} antibody positive. The mean index value of GAD_{65} positive individuals was 0.83 (range: 0.08–2.42). No difference in antibody reactivity against CVB4 proteins was observed in the GAD_{65} antibody-positive and GAD_{65} antibody-negative serum samples (Fig. 3).

Antibody Reactivity Against p2C

Antibody reactivity against p2C was confirmed by precipitation of p2C translated in vitro, by which we excluded possible co-precipitation of the viral p2C with other viral proteins. Antibodies against p2C were detectable in all sera from diabetes patients and siblings,

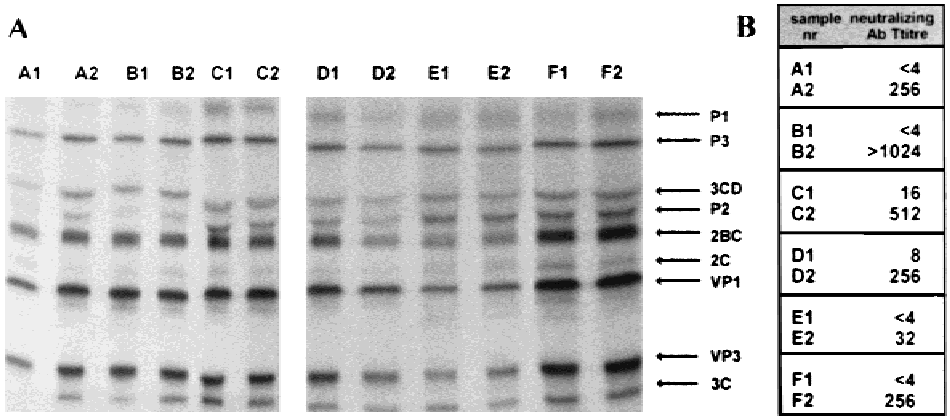

Fig. 2. Immunoprecipitation of coxsackie B4 virus (CVB4) proteins by paired serum samples from subjects experiencing a recent enterovirus infection. **(A)** Immunoprecipitation of CVB4 proteins by acute (1) and convalescent (2) serum samples from patients with a recent enterovirus infection comprising serotype CVB4 (A), CVB3 (**B, C, E,** and **F**), and ECHO virus 20 (EV20) **(D). (B)** The titre of neutralising antibodies for each serum sample is presented. No difference in overall pattern or intensity of the precipitated proteins between the convalescent and the acute samples was observed.

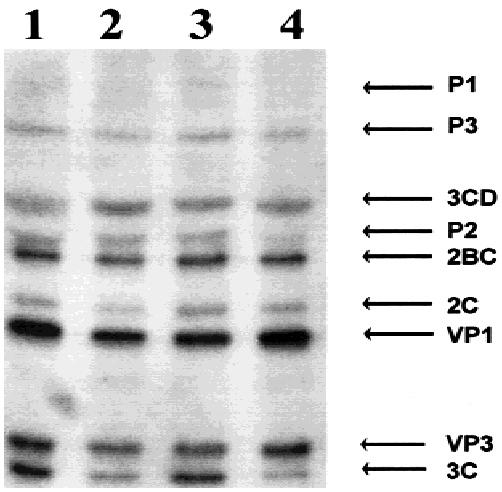

Fig. 3. Antibody reactivity against coxsackie B4 virus (CVB4) proteins in samples from type 1 diabetes mellitus patients and their siblings. Antibody reactivity against CVB4 proteins was detected in sera obtained from type 1 diabetes patients and their siblings as shown by representative data from: a glutamic acid decarboxylase (GAD_{65})-negative sibling (**lane 1**); GAD_{65}-positive sample from a type 1 diabetes patient (**lane 2**); and a GAD_{65}-negative diabetes serum sample (**lane 3**). As a control, precipitation by a CVB3-infected patient is shown in **lane 4**.

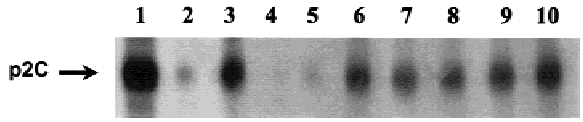

Fig. 4. Antibody reactivity against protein 2C (p2C). In vitro translated p2C was immunoprecipitated by sera from different subjects. In **lane 1** in vitro translated p2C is shown, indicated by an arrow. Immunoprecipitation by serum from a pathogen free rabbit (**lane 2**), rabbit anti-p2C antiserum (**lane 3**), rabbit anti-p2B antiserum (**lane 4**), and precipitation in the absence of serum (**lane 5**) are included as either negative or positive controls. Representative results of anti-p2C positivity in glutamic acid decarboxylase (GAD_{65})-positive (**lane 6**), and GAD_{65}-negative sera (**lane 7**) are shown. Sera from enterovirus patients ((CVB4), **lane 8**; (EV11), **lane 9**) and healthy donors (**lane 10**) were positive for antibodies against p2C.

either GAD_{65} antibody positive or negative (Fig. 4). Sera from nondiabetic subjects with a recent enterovirus infection and sera from healthy subjects did not contain antibodies against GAD_{65}, but showed antibody reactivity against p2C (Fig. 4).

DISCUSSION

The finding that antibodies against nonstructural enterovirus proteins are highly prevalent in the population, both in patients with a recent infection and healthy donors, is not unexpected. The high antibody prevalence corresponds with the high frequency of enterovirus infections in the population, most frequently causing the so-called "summer-flu" or inducing subclinical infections [Melnick, 1996]. Antibodies against nonstructural proteins, however, have been documented only rarely and possible cross-reactivity between nonstructural proteins of different but related enteroviruses has not been studied extensively [Ehrenfeld et al., 1995]. We observed that serum samples obtained from patients with different enterovirus infections reacted with CVB4 antibodies, which suggests a previous infection with the CVB4 serotype. However, most probably these antibodies are induced by other, related serotypes. Based on the relatively conserved sequences of nonstructural enterovirus proteins [Holland et al., 1982; Zoll et al., 1994], a high degree of heterotypic cross-reactivity can be expected. Indeed, the results strongly suggest the existence of a very broad heterotypic cross-reactivity. CVB4 proteins were precipitated irrespective of the enterovirus strain by which the subjects were infected. Moreover, the sera did not differ in their capacity to precipitate other enterovirus strains, for example, CVB3 and poliovirus.

The role of antibodies against nonstructural proteins in the defence against viral infections is not clear. These antibodies might be secondary to virally induced cell lysis rather than be involved in the clearance of the infection. Because nonstructural proteins are exclu-

sively produced during viral replication, induction of antibodies against nonstructural proteins is dependent on active replication of the virus. That makes these antibodies attractive components to discriminate between immune responses induced by wild-type infection or by vaccination with killed virus, for example, against poliovirus [Ehrenfeld et al., 1995]. Such a discrimination would be of value in the monitoring of circulating poliovirus in the population and the prevalence of subclinical poliovirus infections, which are important issues in the follow up of the poliovirus eradication programme. Whereas none of the subjects had a clinical history of infection with poliovirus, and most of the individuals were vaccinated with inactivated (nonreplicating) poliovirus vaccine, poliovirus nonstructural proteins were precipitated by all of the sera (data not shown), indicating that antibodies against enteroviral proteins cross-react with the poliovirus nonstructural proteins. The high degree of antibody cross-reactivity against nonstructural proteins limits the possibility to use antibodies against nonstructural proteins as a marker of a natural poliovirus infection.

The sequence homology between p2C and GAD_{65}, the major autoantigen in type 1 diabetes mellitus has been proposed as a target of immunological cross-reactive responses. Although it has been shown that cross-reactivity against GAD_{65} and p2C is possible, the role in the pathogenesis of type 1 diabetes mellitus is not known and there is no consensus whether significant differences in p2C antibody reactivity exists between diabetes patients and healthy controls [Hou et al., 1994; Lönnrot et al., 1996]. Deletion mutants of GAD_{65} demonstrated that the region of similarity between GAD_{65} and p2C is involved in recognition of GAD_{65} by GAD_{65}-antibodies [Richter et al., 1994]. However, monoclonal antibodies against this region, obtained from type 1 diabetes mellitus patients, did not cross-react with p2C [Richter et al., 1994], suggesting that there is no involvement of antibody mediated mimicry in patients with type 1 diabetes mellitus.

It has been reported that antibodies against GAD_{65} are directed mainly against conformational epitopes [Richter et al., 1994]. Therefore, studies investigating antibody cross-reactivity with synthetic peptides must be considered with caution. In our study, we used native p2C, translated and processed by virally infected cells, which closely resembles the in vivo situation. Therefore, it was possible to detect all conformational and linear epitopes as expressed naturally in the in vivo situation. The p2C antibodies were detected in all of the serum samples tested, either GAD_{65} antibody positive or GAD_{65} antibody negative. The high antibody prevalence does not agree with the observation by Richter et al. [1994], who detected p2C antibodies in only 3 of 10 serum samples from patients and healthy control donors. The high anti-p2C antibody prevalence correlates well with the observed reactivity against other nonstructural proteins of enteroviruses. GAD_{65} antibodies were not detectable in healthy controls or subjects with a recent enterovirus infection, although p2C antibody positive, indicating that reactivity against CVB4 p2C does not necessarily induce a cross-reactive immune response with GAD_{65}. As a consequence of the high prevalence of anti-p2C reactivity, a correlation between p2C antibodies and GAD_{65} antibodies cannot be established.

Several remarks have to be made concerning the lack of association between GAD_{65} and p2C antibody reactivity and the relevance of molecular mimicry for the development of type I diabetes mellitus. First, based on a genetic predisposition, subjects at risk of development of type 1 diabetes mellitus may direct their immune response to different CVB4 p2C epitopes (e.g., the PEVKEK region) as compared with subjects not at risk for the disease. It has to be determined whether in type 1 diabetes mellitus patients anti-p2C antibodies are directed against the homologous region. As yet, antibodies against the homologous region of GAD_{65} obtained from type 1 diabetes mellitus patients have not been shown to cross-react with p2C [Richter et al., 1994]. Second, animal experiments have shown that a high variety in diabetogenicity of different CVB strains exists, probably due to differences in tissue tropism of the virus [Kuno et al., 1984; Szopa et al., 1990; Ohashi et al., 1993]. By testing antibodies against viral proteins, we cannot distinguish between diabetogenic and nondiabetogenic variants. A combination of several viral factors, for example, the frequency and localisation of coxsackie B viral infections, and the antigenic determinants of the virus are factors that may all contribute to the development of type I diabetes mellitus [Katz et al., 1995; Ramsingh et al., 1997]. Third, antibody responses against GAD_{65} may be secondary to beta-cell destruction rather than being initiators of autoimmune destruction of the insulin producing cells [Petersen et al., 1993]. The cellular immune response might be more relevant in the pathogenesis of type 1 diabetes mellitus than antibody-mediated molecular mimicry [Rossini et al., 1993].

In conclusion, the results demonstrate that antibody reactivity against p2C is highly prevalent in the population and does not generally cross-react with GAD_{65}.

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